Fighting for territories: time-lapse analysis of dental pulp and dental follicle stem cells in co-culture reveals specific migratory capabilities

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FIGHTING FOR TERRITORIES: TIME-LAPSE ANALYSIS OF DENTAL PULP AND DENTAL FOLLICLE STEM CELLS IN CO-CULTURE REVEALS SPECIFIC MIGRATORY CAPABILITIES

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Abstract

Stem cell migration is a critical step during the repair of damaged tissues. In order to achieve appropriate cell-based therapies for tooth and periodontal ligament repair it is necessary first to understand the dynamics of tissue-specific stem cell populations such as dental pulp stem cells (DPSC) and dental follicle stem cells (DFSC). Using time-lapse imaging, we analysed migratory and proliferative capabilities of these two human stem cell lines *in vitro*. When cultured alone, both DPSC and DFSC exhibited low and irregular migration profiles. In co-cultures, DFSC, but not DPSC, spectacularly increased their migration activity and velocity. DFSC rapidly surrounded the DPSC, thus resembling the *in vivo* developmental process, where follicle cells encircle both dental epithelium and pulp. Cell morphology was dependent on the culture conditions (mono-culture or co-culture) and changed over time. Regulatory genes involved in dental cell migration and differentiation such as *TWIST1*, *MSX1*, *RUNX2*, *SFRP1* and *ADAM28*, were also evaluated in co-cultures. *MSX1* up-regulation indicates that DPSC and DFSC retain their odontogenic potential. However, DPSC lose their capacity to differentiate into odontoblasts in the presence of DFSC, as suggested by *RUNX2* up-regulation and *TWIST1* down-regulation. In contrast, the unchanged levels of *SFRP1* expression suggest that DFSC retain their potential to form periodontal tissues even in the presence of DPSC. These findings demonstrate that stem cells behave differently according to their environment, retain their genetic memory, and compete with each other to acquire the appropriate territory. Understanding the mechanisms involved in stem cell migration may lead to new therapeutic approaches for tooth repair.

Keywords: Tooth; odontoblast; dental follicle; periodontal ligament; dental pulp; dental stem cells; tooth repair; regeneration.

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Introduction

Cell migration is a widespread, highly dynamic and complex process that is crucial for the appropriate development of organs and tissues (Aman and Piotrowski, 2010; Binamé *et al.*, 2010). Aberrant cell migration often results in severe morphogenetic defects and/or diseases (Friedl and Gilmour, 2009). Reactivation of cell migration underlies tissue repair processes, as well as several pathological conditions, such as metastatic cancers, thus making the study of cell movement clinically relevant.

In order to understand better the mechanisms involved in tissue repair and/or regeneration *in vivo*, *in vitro* assay systems have been developed that can bring considerable information about motility and directional migration of cells (Okumoto, 2010; Stephens and Allan, 2003; Wang *et al.*, 2008). Cellular dynamics and functions (e.g. cell migration, proliferation, apoptosis) can be visualised and quantitatively analysed through computational live image processing (Huth *et al.*, 2011; Wang *et al.*, 2008), thus allowing us to obtain biochemical and biophysical information about different cell populations at precise temporo-spatial windows (Aman and Piotrowski, 2010).

Tooth development results from sequential and reciprocal interactions between the oral epithelium and the cranial neural crest-derived mesenchyme (Bluteau *et al.*, 2008; Mitsiadis and Graf, 2009; Mitsiadis and Luder, 2011; Thesleff *et al.*, 1989; Thesleff *et al.*, 1991; Thesleff *et al.*, 1995). Epithelial cells give rise to the enamel producing ameloblasts, while mesenchymal cells are involved in the formation of the dental pulp and dental follicle. Odontoblasts originated by the dental pulp produce the dentin matrix, whereas dental follicle cells contribute to the formation of cementum, periodontal ligament, and alveolar bone (Diep *et al.*, 2009). Periodontal ligament occupies the space between the tooth root and the alveolar bone and serves for tooth anchorage to the bone and masticatory

force distribution. Although it has been shown that dental follicle cells express a plethora of regulatory molecules (Liu and Wise, 2007; Morsczeck and Schmalz, 2010), the mechanisms controlling their *in vivo* behaviour (e.g., cell proliferation, migration, and differentiation) remains elusive. Dental follicle cells are in close contact with cells of the dental papilla and root dentin during odontogenesis (Mitsiadis and Luder, 2011; Thesleff *et al.*, 1989), and it has been demonstrated that these interactions constitute an important step for dental follicle differentiation (Bai *et al*., 2010). Signalling molecules, derived from the dental papilla and root dentin, could be responsible for the proliferation and differentiation of dental follicle cells *in vivo* (Bai *et al*., 2010; Mitsiadis and Rahiotis, 2004).

During tooth repair after injury or carious lesion, numerous genes that are expressed throughout embryonic tooth development are reactivated. For example, nestin and Notch molecules are re-expressed in the dental pulp during the reparative processes (Mitsiadis and Rahiotis, 2004). Tooth repair also involves activation of various dental stem cell populations (Mitsiadis *et al*., 2011). During tissue repair and/or regeneration, stem cells self-replicate, generate daughter cells and finally re-populate the damaged tissue (Laird *et al*., 2008; Smith, 2001; Smith, 2005). This process requires the oriented or directed movement of stem cells toward this particular anatomic destination (Laird *et al*., 2008). In human teeth, stem cell populations have been isolated and characterised from dental pulp and dental follicle (Gronthos *et al*., 2000; Handa *et al*., 2002; Laino *et al*., 2005; Miura *et al*., 2003; Papaccio *et al.*, 2006; Takeda *et al*., 2008). Dental pulp stem cells (DPSC) and dental follicle stem cells (DFSC) were sorted by FACS using cell-surface markers such as CD117, CD34 and flk-1 for DPSC (d'Aquino *et al.*, 2007; Graziano *et al*., 2008; Tirino *et al*., 2011) and SSEA4, OCT-4, TRA1-80 and TRA1-81 for DFSC (d'Aquino *et al*., 2011). These stem cell populations reside in various niches within the dental pulp and follicle (Lovschall *et al*., 2007; Mitsiadis *et al*., 2011). DPSC are very flexible and can differentiate into chondrocytes, adipocytes, neurons, muscles, odontoblasts and bone cells. *In vivo*, DPSC can form a vascularised pulp-like tissue that is surrounded by odontoblast-like cells (Nakashima and Iohara, 2011). The first clinical trial was successfully performed a few years ago, where DPSC from patients were capable to fully repair their own alveolar bone defects (d'Aquino *et al*., 2009). Similarly to the DPSC, DFSC exhibit a great differentiation potential (e.g. adipocytes, myoblasts, neurons, glial cells, cementoblasts, periodontal ligament fibroblasts), with the exception of bone formation (d'Aquino *et al*., 2011; Morsczeck *et al*., 2005; Yao *et al*., 2008). Indeed, DFSC are not capable of differentiating into osteoblasts unless a specific culture medium is used (Bai *et al*., 2010; d'Aquino *et al*., 2011; Yagyuu *et al.*, 2010; Wu *et al*., 2008). Taken together these findings indicate that DPSC and DFSC may have different functions when used for tissue repair *in vivo*. Thus, it is desirable to understand their behaviour *in vitro* better, before any future clinical application.

In this study we applied time-lapse cell imaging, in an attempt to understand complex biological phenomena related to dental injury and repair/regeneration. We have found that DPSC and DFSC in co-culture behave differently from in mono-culture and compete with each other to increase their vital territory. The fact that DFSC rapidly migrate and occupy most of the culture space by surrounding and restraining the DPSC suggests new criteria for the clinical use of the various stem cell populations during tooth repair.

Materials and Methods

Subjects, stem cell extraction, digestion, and culture

Dental pulps were extracted from intact teeth of 21 to 45 year-old healthy individuals. Dental follicles were collected from wisdom tooth germs with not yet formed roots from 18 to 40 year-old healthy individuals. Dental pulps as well as dental follicles were incubated in a digestive solution composed of 3 mg/mL type I collagenase and 4 mg/mL dispase in phosphate buffered saline (PBS) for 1 h at 37 °C. After enzymatic digestion, the solution was filtered through 70 µm Falcon strainers (Becton & Dickinson, Milan, Italy) and then cells were immersed in DMEM culture medium supplemented with 10 % foetal bovine serum (FBS) (Lonza, Milan, Italy), 100 mM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy) and cultured in 75 cm^2 flasks with filtered valves (Papaccio *et al*., 2006). FGF2 (20 ng/mL) was added to the medium for the culture of the dental follicles. Cells were cultured at 37 °C in a 5 % CO_2 incubator. The culture medium was renewed twice per week. Upon confluence, cells were used either for cell sorting or for time-lapse experiments (generally, at the first passage). Digested tissues were permitted to achieve near confluence (90 % of flask surface), which corresponds to $4x10^5$ cells/25 cm^2 for both cell populations. However, the average doubling time for DPSC is about 3 d while for DFSC it is 1 d (for details see Tirino *et al*., 2011).

Colony efficiency assays and proliferation potential

To evaluate colony efficiency and proliferation potential, single cells obtained by limiting dilutions were plated. After three weeks of culture, cells were stained with 0.1 % (w/v) toluidine blue in 1 % paraformaldehyde (PFA) and the number of clones (>50 cells) was counted.

Fluorescence-activated cell sorting (FACS)

For the collection of DPSC, approximately $1x10^6$ dental pulp cells per sample were detached from the flasks, washed and incubated with the CD117 and CD34 antibodies for 30 min at 4 °C. After incubation, CD34 and CD117 co-expressing cells were sorted using a FACS Aria II BD (BD Biosciences, Milan, Italy). The purity of these sorted cell populations was 90 %. The $CD34^+/CD117^+$cells were cultured in DMEM supplemented with 10 % FBS and then used for the time-lapse experiments.

For the collection of DFSC, nearly $1x10^6$ follicle cells per sample were detached from the flask using 0.02 % EDTA in PBS, pelleted (10 min at 1,000 rpm), washed in 0.1 % bovine serum albumin (BSA) in PBS at 4 ºC, and then incubated with the SSEA4 antibody for 30 min at 4 °C.

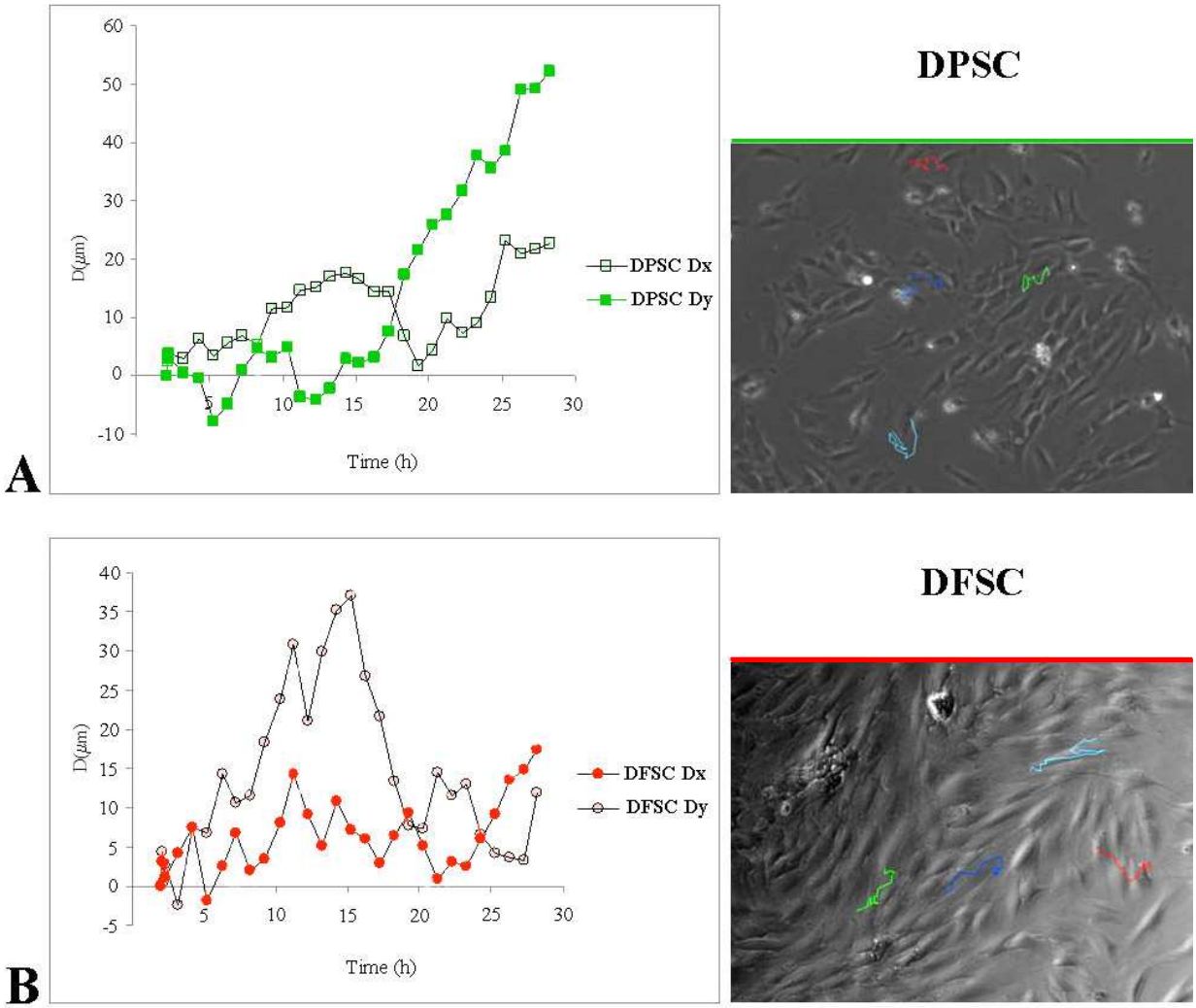

Fig. 1. Cell movement and orientation of DPSC and DFSC cultured alone. (**A**) When cultured alone, DPSC exhibit a random and restricted motility through the x or y axes. Coloured lines (right image) are representative of extracted cell tracks for DPSC in overall time (30 h). (**B**) When cultured alone, DFSC show circular movements. Coloured lines (right image) represent extracted cell tracks for DFSC in overall time (30 h).

After washing, the cells were analysed by flow cytometry and sorted by FACS. The purity of the sorted SSEA4$^+$ population was 90 %. SSEA4$^+$ cells were cultured in DMEM at 10 % FBS and used for time-lapse experiments.

Dye staining

Lipophilic cell tracking dyes such as PKH26 and PKH67 (Sigma Aldrich, Milan, Italy), use membrane-labelling technology to stabilise the incorporation of a fluorescent dye into the lipid regions of the cell membrane. Lipophilic cell labelling is simple to use and allows the follow up of the tagged cells *in vitro* and/or *in vivo*. Therefore, it constitutes a powerful tool for studying the kinetics and behaviour of various cell populations in a heterogeneous environment. DPSC and DFSC were stained with the PKH26 and PKH67 dyes, respectively, at a final concentration of $2x10^{-6}$ M and $3x10^5$ cells/mL according to the manufacturer's instructions. Diluent C is the labelling vehicle provided with the kit, an isosmotic aqueous solution designed to maintain cell viability and maximise dye solubility and staining efficiency (Lee-MacAry *et al*., 2001).

Time-lapse co-culture experiments

DPSC were cultured together with DFSC in a standard 24-well culture plate with μ-dish (35 mm, high) culture-insert (Ibidi, Integrated BioDiagnostics, Munich, Germany). In order to identify the behaviour of these two specific dental stem cell populations in this co-culture system, a series of cell mixtures with different ratio of DPSC/DFSC (i.e.$1.7x10^2$ cells/mm^2/$1.7x10^3$ cells/mm^2{1:10}, $1.7x10^3$ cells/mm^2/$1.7x10^2$ cells/mm^2 {10:1}, $2.8x10^2$ cells/mm^2/$2.8x10^2$ cells/mm^2{1:1}) were prepared (Fig. 1). Briefly, adherent cells were removed from flasks after 24 h of culture, using proteolytic enzymes (trypsin/EDTA; Invitrogen, Milan, Italy), immediately re-suspended and either plated as such or labelled prior to seeding. The labelling procedure consists in re-suspending centrifuged cells in 1 mL of staining solutions (i.e. PKH26 and PKH67) for 5 min, washing in culture medium for fluorescent dye excess removal, and finally seeding and incubating at 37 °C. After 2 h of incubation, fluorescence microscopy images were taken to analyse the morphology of the cells. The cells were observed for 30 h on Oko-Full time-lapse

in bright field and in fluorescence. In particular, the images in bright field were captured every 1 h (Δt), while those in fluorescence were captured every 4 h. This feature allowed us to reduce sample photo-bleaching and cell damage. It is possible to change the acquisition time of the images through a Ludl shutter controlled by the Oko-Vision software. The latter is an optical video-microscopy station, composed of a microscope (Zeiss Axiovert 200), with a 10x objective in phase contrast, equipped with motorised "stage incubator" for automated sample positioning, a stage incubator where the temperature and the atmosphere were kept at 37 °C and 5 % CO_2, respectively, and controlled humidity. The sequential images were captured by a CCD video camera (ORCA ER, Hamamatsu Photonics, Hamamatsu City, Japan). The monolayer was imaged using phase contrast, while the fluorescently labelled stem cells were imaged using Chroma Filters multi-channel (86013V2), for FITC (excitation BP 450-490 nm, emission LP 515 nm) and TRITC (excitation BP 550 nm, emission LP 580 nm).

Cell tracking software and analysis

The time-lapse video microscopy system represents a novel fully automated high-throughput approach for a precise and detailed cell tracking. In time-lapse video microscopy, manual cell tracking remains the most common method for analysing migratory behaviour of cell populations. Cell-Tracker, automatic tracking and analysis software was implemented using oko-vision (version 2009) and consists of a graphical, cross-platform open source application, adjustable to various types of microscopy images and video files. A modular architecture allows for the expansion of image processing and independent tracking. The Graphicator allows plotting of cell coordinates *versus* time, cell velocity and orientation. Cell-Tracker software allows easy following of cell movement in an interactive way. The cell trajectory is superimposed on the images, for visual validation of the analysis.

Manual tracking was performed with a custom viewing program that enabled storage of x and y coordinates by clicking on cells in sequential images with a computer mouse. Manual cell tracking was performed for a total of 4 cells for each field of view (object), for a total of four objects selected in each well. Generally, each condition was repeated at least three times. In addition, each experiment was performed for all cells located within the preselected regions of analysis, during the considered recording time (i.e. 30 h - 30 tracks).

The speed and motility of DPSC and DFSC in separate wells were analysed using the above-mentioned software. The speed, area, deformation, trajectory and detailed tracking of the cells were computed and displayed for analysis. The interaction of the two dental stem cell populations was studied in different conditions in the same well. Tabulated data of tracking results were exported into Microsoft Excel for further numerical analysis and evaluated statistically using Student's *t*-test. The conversion factor for measured pixels to microns (1.06 for 10x objective) was determined using a stage micrometer.

Cell-Tracker was plotted as cell coordinates (x and y) *versus* time. The cell velocity along x axis (vector) was calculated by the following equation:

$$v_x = \frac{Dx}{t_f - t_0}$$

where Dx denotes the last cell tracker, t_f and t_o represent final time and initial time, respectively.

Analyses were performed considering the ratio DPSC/DFSC ($1.7x10^3$ cells/mm^2/$1.7x10^2$ cells/mm^2 and $1.7x10^2$ cells/mm^2/$1.7x10^3$ cells/mm^2) per well and the major cell number ($1.7x10^3$ cells/mm^2). Data represent the mean ± SD of three independent experiments.

Isolation of total RNA and quantitative real-time (qRT)-PCR

Total RNA was extracted by using 1 mL of TRIzol® (Invitrogen, Milan, Italy), according to the manufacturer's instructions. The precipitate was then re-suspended in nuclease-free water. The concentration of the extracted RNA was determined using a Nanodrop spectrophotometer (Celbio, Milan, Italy), and qualitative analysis of the RNA was accomplished by 1 % agarose-gel (w/v) electrophoresis. For cDNA synthesis, performed with the Reverse Transcription System Kit (Promega, Milan, Italy), 1 μg of DNase-digested total RNA was used (DNA-free kit; Ambion-Applied Biosystems, Monza, Italy). Quantitative RT-PCR was obtained by using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Milan, Italy) to analyse the expression of *TWIST1*, *MSX1*, *RUNX2*, *ADAM28* and *SFRP1*. BLAST query permitted the specificity analysis of

Table 1. Oligonucleotide primers for real time PCR.

Primers	Sequence	Gene Function	T °C Annealing
TWIST-1	Sense GGCACCATCCTCACACCTCTG Antisense TGGCTGATTGGCACGACCTC	Cell lineage determination, cell differentiation	57 °C
MSX-1	Sense ACTGAGACGCAGGTGAAGATATGG Antisense CCGCCGAGAGGGAAGGAGAG	Craniofacial development, odontogenesis	55 °C
RUNX-2	Sense ACCAGCAGCACTCCATATCTCTAC Antisense CTTCCATCAGCGTCAACACCATC	Osteoblastic differentiation, skeletal morphogenesis	55 °C
SFRP-1	Sense TGTAATCCAGTCGGCTTGTTCTTG Antisense GGCTGCTGCTCCACATTGC	Regulation in cancer, dental follicle development	55 °C
ADAM-28	Sense TTGTGGTGGTTGCTATGGTAATCC Antisense GGCTTCATCTGACTCATCTCTTGG	Cell-cell and cell-matrix interactions.	56 °C

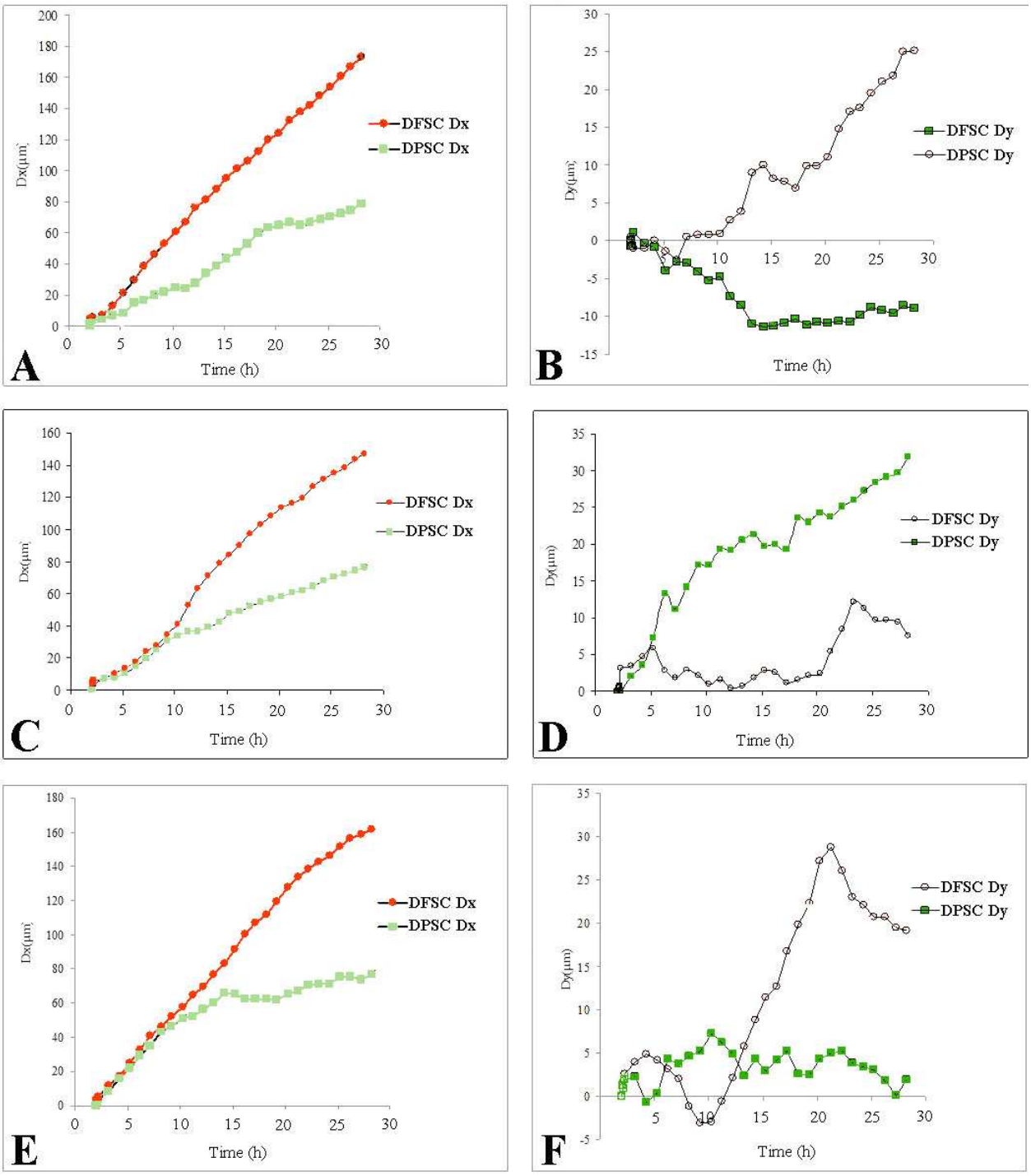

Fig. 2. Cell motility in co-culture of DPSC and DFSC. The graphs show the cell tracker analyses of DPSC and DFSC co-cultured at a ratio 1:1 along x **(A)** and y axis **(B)**, at a ratio 10:1 along x **(C)** and y axis **(D)**, and at ratio 1:10 along x (**E**) and y axis (**F**).

each qRT-PCR primer pair, and corresponding sequences were designed by Beacon Designer™ software. The primer sequences are shown in Table 1. All reactions were carried out in triplicate, and the expression of specific mRNA relative to the control was determined after normalisation with respect to GAPDH gene. The fold-change of test gene mRNA expression was calculated by considering the efficiency of each primer (between 80 and 110 %), and by using the comparative threshold method (ΔΔCt = difference of ΔCt between co-cultured cells and single cell populations used as controls). The results were expressed as normalised fold expression, calculated by the ratio of crossing points of amplification curves of several genes and internal standard, by using the Bio-Rad iQ™5 software (Bio-Rad Laboratories Srl).

Results

Colony efficiency assays and proliferation potential

To assess the proliferation and clonogenic potential of DPSC and DFSC, we performed a limiting dilution

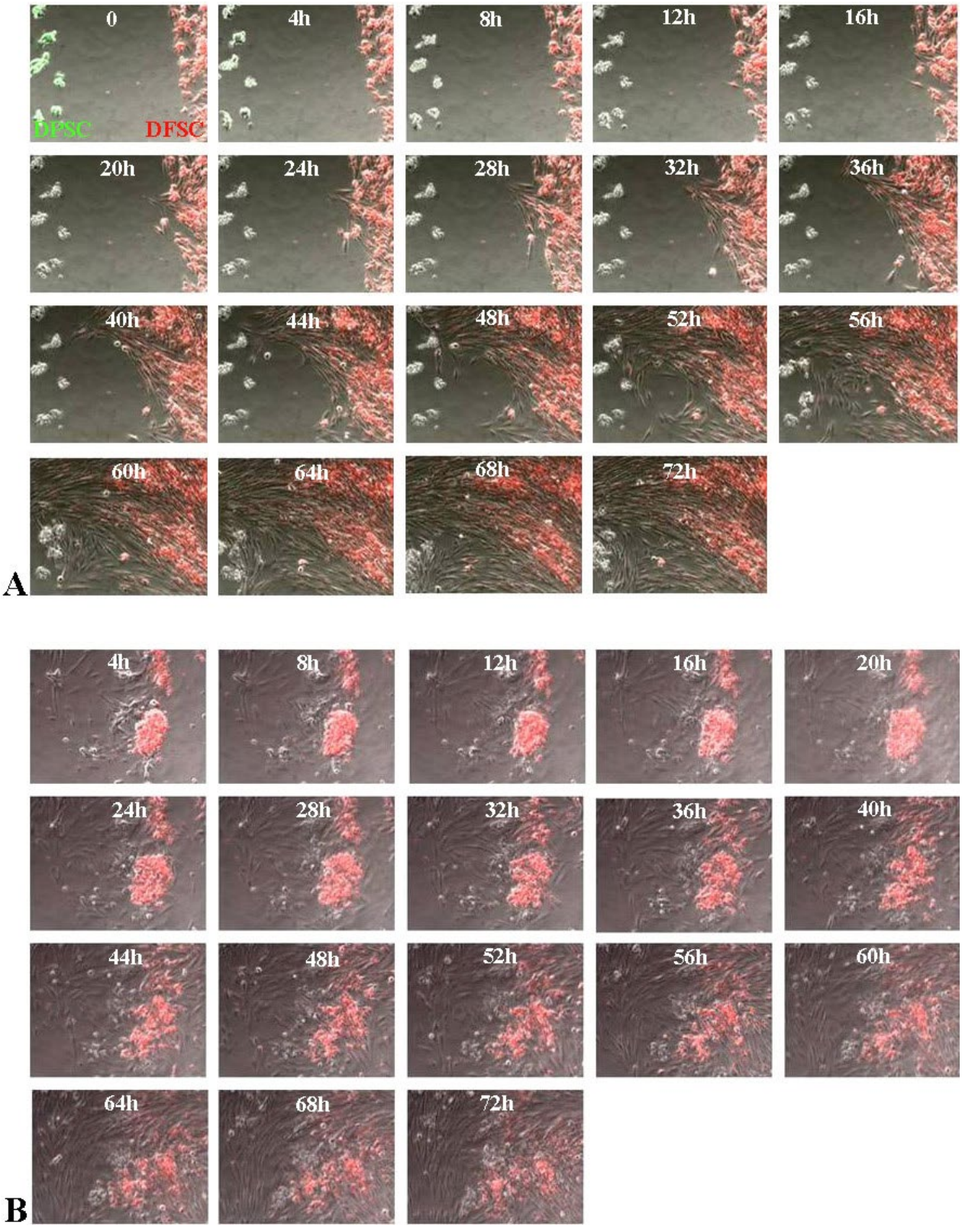

Fig. 3. Time-lapse panel images. (**A**) DPSC and DFSC in co-culture at a ratio $1.7x10^2$ cells/mm^2/$1.7x10^3$ cells/mm^2 (1:10). Note that DFSC completely surround DPSC. (**B**) DPSC and DFSC in co-culture at a ratio $1.7x10^2$ cells/mm^2/$1.7x10^2$ cells/mm^2 (1:1). Note that DPSC do not move, building up a wall slide by slide. Merge micrographs (bright field, FITC and TRITC) captured at 4 h intervals.

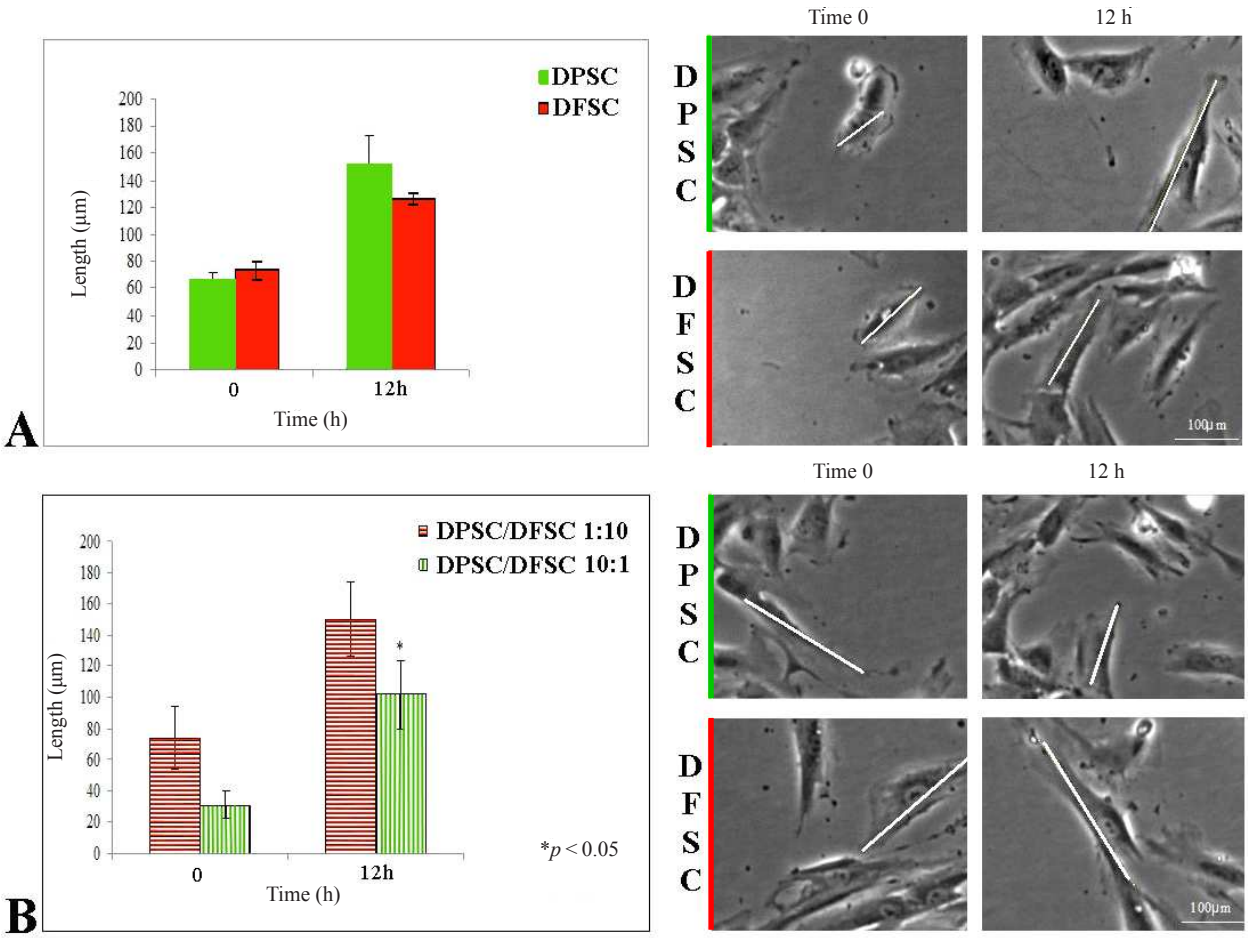

Fig. 4. Cell dimension analysis. (**A**) Major axis averaged sizes during time of DPSC and DFSC cultured alone, demonstrating that in both populations cells become elongated. (**B**) Major axis averaged dimensions during time of DPSC and DFSC co-cultured at a ratio 1:10 and 10:1. In both cases DFSC significantly ($p < 0.05$) increase their sizes, whereas DPSC shorten them.

assay. After 3 weeks of culture, 92 % of the wells (442 of 480), which were initially plated with one or two DPSC, contained colonies (formed of >50 cells), with a doubling time of ≈2.6 d. For DFSC, 93 % of the wells (445 of 480) contained colonies with a doubling time of ≈2.4 d.

Cytometry assay

To isolate DPSC, dental pulp cells were detected and sorted for co-expression of CD34 and CD117 markers at passage 1 of culture. The positivity of these antigens was ~15 % of the total cell population. To isolate DFSC, dental follicle cells were detected and sorted for SSEA4 expression at passage 1 of culture. The percentage of SSEA4 positivity was ~80 %. DPSC and DFSC were sorted and then used for time-lapse and RT-PCR experiments.

Time-lapse observations

In order to observe the behaviour of DPSC and DFSC *in vitro* (i.e. cell movement, orientation, morphology and velocity), time-lapse video-microscopy analyses were performed on 6-8 tracks for each image (4 fields of view per well). These two stem cell populations were cultured either alone or together in various cell ratios. As a control, $1x10^4$ DPSC or DFSC per well (200 mm^2) were used. In co-cultures, DPSC and DFSC were seeded separately using a spacer (Culture-Insert). In a first set of experiments, the cell ratio was fixed at 1:1, 1:10 and 10:1 along x and y axes, and each stem cell population was stained with a different dye (green colour for DPSC, red colour for DFSC). Analyses of the DPSC and DFSC behaviour were performed on the side of the well containing the greater number of cells ($1.7x10^3$ cells/mm^2).

Cell movement and orientation

When cultured alone, DPSC and DFSC moved trivially for short distances and often returned in their point of origin (Fig. 1A and B). More precisely, DPSC showed random and small motility through the x or y axes (Fig. 1A), while most of the DFSC displayed circular movements within a small perimeter (Fig. 1B). The behaviour of DPSC and DFSC changed significantly in the co-culture experiments, and this was dependent on both the number and ratio of cultured cells.

DPSC/DFSC 1:1 co-culture

A substantial and interesting change of cell movements was observed when the two stem cell populations were seeded at the same density. In this case, a better linear progression was detected for DFSC movements along both coordinates (x and y axis), as confirmed by curve analyses, although

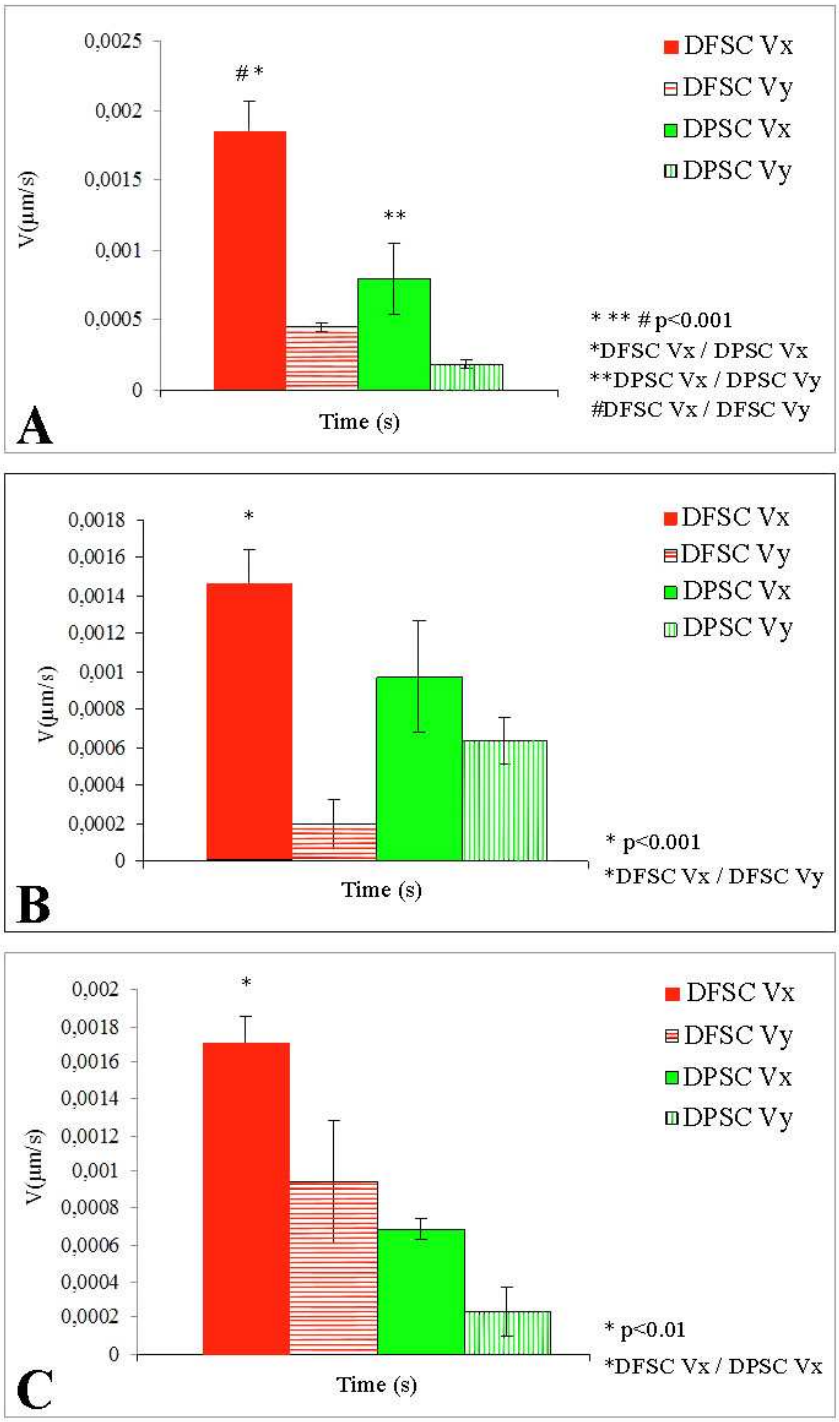

Fig. 5. Cell velocity analysis. DPSC and DFSC in co-culture at a ratio 1:10 (**A**), 10:1 (**B**), and 1:1 (**C**). DFSC result in 2-fold horizontal velocity in comparison with DPSC and even 3-fold along the vertical axis. Data represent the mean ± SD of three independent experiments. The groups are significantly different according to Student's *t*-test ($p < 0.001$).

DFSC moved oppositely to DPSC along the y axis. The track of DPSC along the x axis (Dx) was more linear than the track along the y axis (Dy) (Fig. 2A and B).

DPSC/DFSC 10:1 co-culture

When DPSC and DFSC were co-cultured at a 10:1 ratio, both cell populations exhibited a linear progression along the x axis (Dx), while cell movements were highly irregular along the y axis. More precisely, DPSC showed only short irregular movements, and remained practically in the same position. In contrast, DFSC covered a long distance, and although their initial movement was irregular it finally became linear and unidirectional (Fig. 2C and D).

DPSC/DFSC 1:10 co-culture

When DPSC and DFSC were co-cultured at the 1:10 ratio, both cell types showed a linear progression of their movements along the x axis (Dx), while their movements were irregular along the y axis (Dy). However, DFSC moved more than DPSC along the y axis (Fig. 2E and F).

Image analyses

The observation of the panels (Fig. 3) confirmed that there is an interaction between DFSC and DPSC in terms of motility. We observed that DFSC movement was mainly directed along the horizontal axis on the way to surround the DPSC, almost embracing or moving them out, occupying the whole territory in a short period of time (Fig. 3A). In contrast, the movement of DPSC was comparable to a plug flow along the x axis, being much slower than that of DFSC. The images clearly showed that DPSC migration was less directional than the DFSC migration (Fig. 3B). At the initial stages of the co-culture (day 1), we have observed "pioneer" DFSC moving towards DPSC (Fig. 3A). These "pioneer cells" quickly returned to the DFSC territory once they approached or contacted the DPSC. This step may represent a stimulatory signal to the whole DFSC population for starting their migration towards DPSC (see also supplementary movies 1, 2 and 3 on the kinetics of DFSC "red cells" and DPSC "green cells" – on the web page for this paper).

Cell morphology

Another important point that has been addressed in this study concerns the morphological modifications (i.e. shapes and dimensions) of DPSC and DFSC when cultured either alone or together. We have observed that, when cultured alone, both DPSC and DFSC (which are slightly longer than the DPSC at the starting point) changed their shapes during movement and became considerably flattened and elongated after 12 h (Fig. 4A). More precisely, cells increased their length by up to 1.7-fold (Table 2). In all co-culture variants, DFSC significantly ($p < 0.05$) increased their size (2-fold higher; Table 2), became elongated and acquired a spindle shape (Fig. 4B). The enveloping attitude of DFSC seemed to induce this restricted elongation of DPSC in co-culture: DPSC decreased in size ($p < 0.05$) and assumed a polygonal shape (Fig. 4B).

Cell velocity

Concerning cell velocity, in co-cultures of DPSC and DFSC at a ratio 1:10, DFSC were twice as fast as DPSC ($p < 0.001$) on the x axis. On the y axis, cell movement was slower when compared with the x axis but also, in this case, DFSC were faster than DPSC (Fig. 5A). In co-cultures of DPSC and DFSC at a ratio 10:1, DPSC v_y was greater than DFSC v_y (Fig. 5B), while at a ratio 1:1, DFSC were significantly faster than DPSC, in both x and y axes (Fig. 5C). Specifically, DFSC v_x resulted in a 2-fold increase over DPSC horizontal velocity, and even 3-fold along the vertical axis (Fig. 5C). This confirms that DFSC are more active when compared with DPSC.

Real-time PCR observations

The expression of several genes that are regulators of cell proliferation, migration and differentiation during odontogenesis was evaluated by RT-PCR in our culture system (Table 1). We have used the comparative threshold method (ΔΔCt = difference of ΔCt between co-cultured cells and single cell populations used as controls) and the results were expressed as normalised fold expression, calculated by the ratio of crossing points of amplification curves of several genes and internal standard. *TWIST1* was markedly up-regulated in DPSC at 24 h, while it was down-regulated at 48 h (Fig. 6A). In contrast, *MSX1* and *RUNX2* expression showed a strong down-regulation at 24 h that changed into a significant up-regulation at 48 h (Fig. 6A). The expression of *SFRP1* was up-regulated at 24 h and 48 h of culture (Fig. 6A). *ADAM28* expression was drastically up-regulated in DPSC after 48 h of culture (Fig. 6A).

Analysis of the same profiles of gene expression in co-cultures with prevalent DFSC has shown that the expression of *TWIST1* was slightly decreased at 24 h and 48 h of culture, whereas expression of *MSX1* and *ADAM28* were significantly up-regulated after 48 h of culture (Fig. 6B). *RUNX2* expression was initially decreased (24 h) and thereafter increased (48 h). *SFRP1* expression was slightly decreased after 48 h of culture.

Discussion

Cell-based dental tissue repair or regeneration is an attractive approach that complements traditional restorative and surgical techniques for replacement of injured or pathologically damaged tissues. Such therapeutic approaches often require large numbers of stem cells that after injection migrate towards the injury site following a gradient of directional stimuli. However, a frequent problem of these therapies is the integration of the injected stem cells with the injured or pathological site (Mitsiadis *et al.,* 2012). A deeper appreciation of the mechanisms involved in stem cell behaviour will certainly facilitate cell-based treatments for tissue repair.

In this study, we investigated the *in vitro* behaviour of two different dental stem cell populations, DPSC and DFSC, using time-lapse imaging. This technique offers an ideal platform for understanding stem cell kinetics in response to injuries and cell-based therapeutic interventions (Aman and Piotrowski, 2010; Wang *et al*., 2008). The ability of DPSC and DFSC to actively migrate, either randomly or directionally, and fill the empty space during dental tissue repair is an important biological parameter in pathological contexts (Huth *et al*., 2011). The vast majority of DPSC and DFSC did not show any peculiar or systematic migratory behaviour when cultured separately *in vitro*. Both DPSC and DFSC exhibit tumbling phases: DPSC showed a limited and random migratory activity, while DFSC followed a circular mode of movement to explore their local environment and often returned to their

Table 2. DPSC and DFSC dimensions when cultured either alone or together.

Major axis length	**T = 0**	**T = 12** h
DPSC	67.1 ± 4.1	151.5 ± 21.7
DFSC	73.6 ± 7.3	126.3 ± 4.4
DPSC/DFSC 10:1	67.1 ± 8.7	87.8 ± 8.2
DPSC/DFSC 1:10	73.7 ± 20.1	150.0 ± 23.1

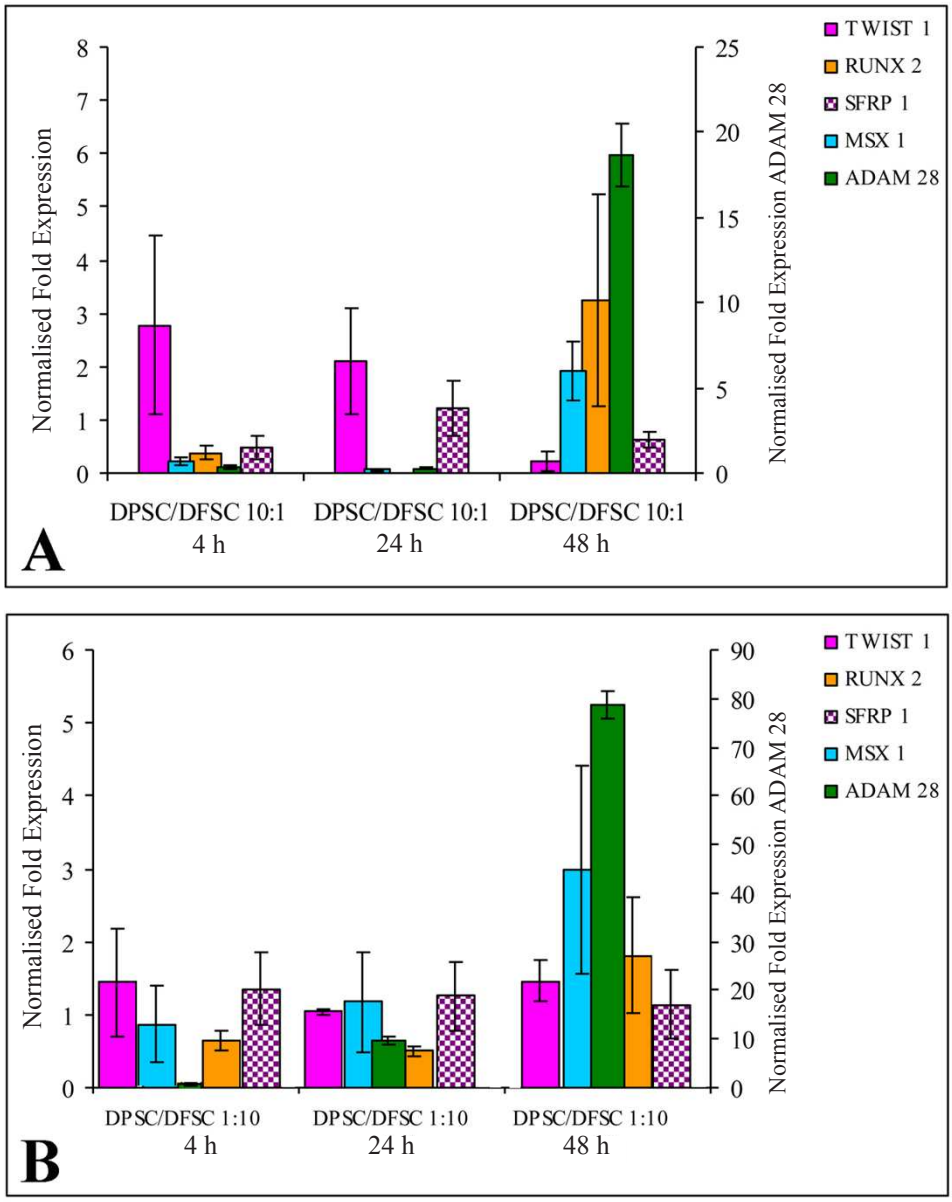

Fig. 6. Gene expression analysis. Images showing normalised fold expression for the genes *TWIST 1*, *MSX 1*, *RUNX 2, SFRP1* and *ADAM 28* in co-cultures of DPSC and DFSC containing (**A**) a prevalent population of DPSC (DPSC/DFSC at a ratio 10:1) and (**B**) a prevalent population of DFSC (DPSC/DFSC at a ratio 1:10).

point of origin. It has been shown that the tumbling phase is cell-autonomous and independent of directional cues such as chemokine signalling (Reichman-Fried *et al*., 2004). DPSC and DFSC motion was tortuous, probably due to the frequent reversals in directions caused by successive protrusions with opposing orientations. Reduction of direction reversals makes cell movement less tortuous and cells adopt an oriented trajectory towards a chemical or electric signal (Aman and Piotrowski, 2010; Arocena *et al*., 2010; Zhao *et al*., 2011a). Indeed, the co-culture of DPSC and DFSC stimulated their migration abilities, particularly when these two stem cell populations were seeded in equal cell numbers. DPSC showed limited proliferation and migration capabilities, and adopted a non-directed random "walk". In contrast, DFSC exhibited quick spreading and directionally migrated towards DPSC. Pre-migratory DFSC started to extend protrusions for guidance and traction in a non-directed fashion. Early DFSC migration serves to populate the empty Petri-dish space, whereas late migration apparently relies on directional cues emanating from DPSC. It has been shown that the direction of late cell migration is established by gradients of repulsive and/or attractant molecules diffusing from the target tissue (Aman and Piotrowski, 2010; Kuriyama and Mayor, 2008). In addition to guidance cues, cell movement requires physical forces that are established through interactions of cells with their environment (Grashoff *et al*., 2010; Hoffman *et al*., 2011). In collectively migrating cells, only a few pioneer (or leading) cells perceive guidance cues (Aman and Piotrowski, 2010; Binamé *et al*., 2010; Friedl and Gilmour, 2009). Indeed, in our co-culture system DFSC started to migrate collectively after getting spatial and directional information from pioneer cells. Migration of pioneer DFSC initially occurred with no interactions with

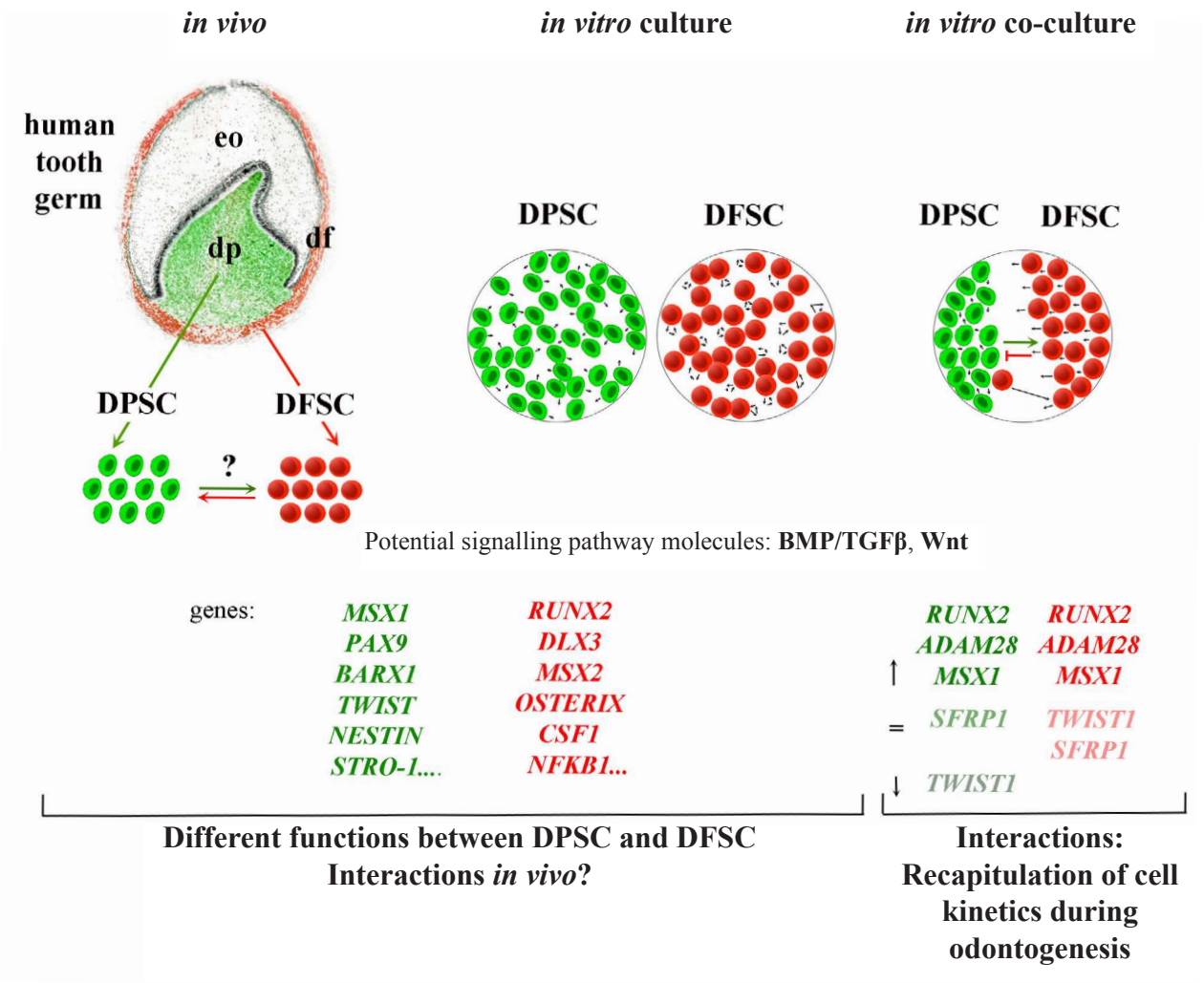

Fig. 7. Schematic representation showing DPSC and DFSC cultured alone or together *in vitro*. DPSC are isolated from the dental pulp (dp, green colour) and DFSC from the dental follicle (df, red colour), which surrounds both the enamel organ (eo) and dental pulp of the developing tooth. *In vivo*, the functions of these two stem cell populations are tissue specific, DPSC control repair/regeneration of the dentin-pulp complex, while DFSC operate for the homeostasis and repair of the periodontium. Genes are differentially expressed in dental pulp (e.g. *MSX1, PAX9* etc., in green) and dental follicle cells (*DLX3, MSX2* etc., in red). Signalling pathways such as BMP/TGFβ and Wnt regulate the expression of these genes. It is not yet known if there are interactions between DPSC and DFSC *in vivo* after tooth eruption. However, these two stem cell populations interact in co-cultures: DFSC migrate fast (small arrows) towards DPSC (probably due to DPSC-derived attractant signals, green arrow) and finally embrace them. In contrast, DPSC stay on place (possibly because of DFSC-derived repulsive signals, red symbol). This movement recapitulates the kinetics of the dental follicle and dental papilla cells during odontogenesis. Expression of several genes was up-regulated (up looking arrow), unchanged (=), or down-regulated (down looking arrow) in co-cultured DPSC and DFSC.

the trailing DFSC. These pioneer DFSC travel until they reach the DPSC territory, and immediately after contacting DPSC they move back to their point of origin where they transmit the acquired information for an oriented DFSC migration towards DPSC. Shortly afterwards, the trailing DFSC migrate fast as cohorts, occupy most of the empty territory, and finally embrace and restrain the DPSC. This DFSC motion closely resembles that of dental follicle cells during odontogenesis, where they encircle the enamel organ and the dental papilla of the developing tooth germ (Fig. 7). Thus, a step of embryonic tooth development was reiterated in the *in vitro* co-culture model. The fact that DFSC are isolated from a developing and not yet differentiated tissue that surrounds the not erupted tooth germ might explain the greater migration activity of DFSC compared with DPSC, which are isolated from the mature pulp tissue. Although collectively migrating cells often lose their motility as they reach their target (Aman and Piotrowski, 2010), DFSC at the end of the migratory pathway were still motile.

Tooth damage is the result of different mechanisms of injury combined with the incapacity for intrinsic dental tissue repair. In autologous stem cell-based approaches, patient-related factors, such as the healthy or pathological condition of the dental pulp and periodontal ligament, may influence the quality of the therapeutic preparation

(Catón *et al*., 2011). Regeneration of dental pulp does not yet constitute a treatment modality in the clinical field of endodontics. The use of DPSC holds a strong promise in this respect. These cells can differentiate into odontoblasts that will form new dentin, endothelial cells that would support the re-vascularisation and neurons that will re-innervate the regenerated pulp tissue (Catón *et al*., 2011; Nakashima and Iohara, 2011). If DPSC are injected into the injured area they do not have to migrate fast or over long distances since the pulp space is delimited by the dental mineral structures, thus making DPSC the most appropriate choice for pulp regeneration. Another major challenge in dentistry is the regeneration of the disease-affected periodontal tissues (i.e. tooth root cementum, alveolar bone and periodontal ligament), in a manner that recapitulates embryonic tooth development. Periodontium acts as a suspension for the tooth, adapting to the mechanical and masticatory loads. DFSC are capable of differentiating into all cell types composing the periodontal tissues, they can travel fast and far away from the site of injection, thus ensuring a quick and appropriate tissue recovery.

To improve the use of DPSC for dentin repair and DFSC for periodontal regeneration, it is important to replicate the permissive signals that initiate the differentiation of pulp cells into odontoblasts and of follicle cells into periodontal ligament fibroblasts, respectively (Fig. 7). Such a strategy is likely to restore better the damaged soft and hard dental tissues. WNT proteins are regulators of cell proliferation, migration and differentiation during tooth development and regeneration (Dassule and McMahon, 1998; Sarkar and Sharpe, 2000), and have a great therapeutic potential for hard tissue remodelling and regeneration after injury (Long, 2011; Reya and Clevers, 2005). Recent clinical therapeutic strategies focus on the inhibition of the WNT antagonists such as the Secreted-Frizzled Related Protein-1 (SFRP1), which is expressed in dental follicle cells during odontogenesis (Liu and Wise, 2007; Liu *et al*., 2012; Morsczeck and Schmalz, 2010). *SFRP1* expression is regulated by TGFβ/BMP signalling molecules (Li *et al*., 2011a) that are involved in dental cell specification and differentiation (Huang *et al*., 2010; Ko *et al*., 2007; Mitsiadis and Graf, 2009; Thesleff *et al*., 1995). For example, aberrant BMP signalling affects odontoblast differentiation and induces ectopic bone formation that replaces normal dentin (Li *et al*., 2011b). In dental mesenchyme, BMP4 regulates the expression of *MSX1* (Vainio *et al*., 1993), which is a crucial gene for tooth formation since its point mutation in humans causes partial anodontia (Vastardis *et al*., 1996). The interplay between TGFβ/BMP and WNT signalling pathways is needed to ensure dental stem cell specification during tooth repair (Du *et al*., 2012; Silvério *et al*., 2012). In our co-culture model, expression of *MSX1* was significantly up-regulated in both DFSC and DPSC, indicating that these two stem cell populations have a great capacity to form dental structures. TWIST1 could promote the odontogenic potential of DPSC by antagonising the function of RUNX2 that favours osteoblast differentiation (Li *et al*., 2011b; Pan *et al*., 2010; Zhao *et al*., 2005). However, the co-culture of DPSC and DFSC resulted in down-regulation of *TWIST1* and significant up-regulation of *RUNX2* expression, suggesting that DPSC lose their capacity to differentiate into odontoblasts in the presence of DFSC. *SFRP1* expression in DFSC was not altered when DFSC were co-cultured with various ratios of DPSC, indicating that DFSC retain their potential to form periodontal tissues in the presence of DPSC. Recent studies have shown that *ADAM28* overexpression has opposite effects on DPSC and DFSC: ADAM28 promotes proliferation of DFSC (Zhao *et al*., 2010), while it inhibits proliferation and induces differentiation of DPSC (Zhao *et al*., 2011b). In co-cultures, *ADAM28* expression was increased dramatically in both DFSC and DPSC, indicating that the proliferative potential resides within DFSC.

Conclusions

In conclusion, our observations have highlighted that DFSC and DPSC behave differently in co-culture from when cultured alone and compete with each other in order to replenish the free territory. The fact that DFSC migrate faster than DPSC and dominate them establishes new criteria for the selection and use of specific stem cell populations for the repair and regeneration of particular dental tissues in the clinic. These results, based on dental stem cell lines, could be extrapolated for the controlled regeneration of organs that have important vital functions.

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Discussion with Reviewers

Reviewer I: As the authors are experts in the dental field, could they discuss the possibility of using mixed populations of DPSC and DFSC in transplantations targeting tooth repair?

Authors: Dental follicle cells and dental papilla cells are in close contact during odontogenesis. Signalling molecules from the dental papilla could promote proliferation and differentiation of dental follicle cells during development. Indeed, in our co-culture model we have shown that DPSC stimulated the migration abilities of DFSC. In addition, previous results have shown that, in co-culture, DPSC can promote differentiation of DFSC and formation of hard tissues (Bai *et al*., 2010). We hypothesise that the use of mixed DFSC and DPSC populations for tooth repair may accelerate cytodifferentiation and hard matrix deposition

events. However, it is of importance to carefully define the appropriate percentage and dosage of DFSC and DPSC according to the targeted dental tissue (dental pulp or periodontal tissues). Taking these parameters into account, repair of the dentin-pulp complex and/or periodontal tissues could occur in a faster than normal way.

In the co-culture, DFSC motion closely resembles that of dental follicle cells during odontogenesis. Dental follicle cells encircle the enamel organ and the dental papilla of the developing tooth germ. DFSC could also be used for the regeneration of a follicle around a tooth germ formed *in vitro* (recombination of dental epithelium with dental papilla mesenchyme: formation of a brand new tooth). Once the follicle will form around the tooth germ, the explant could be implanted into the alveolar bone *in vivo*. This technique will probably allow the development and eruption of a new tooth with all appropriate tissues and possible functions.

Reviewer II: DPSC and DFSC seem to be both originated from mesenchymal stem cells because they share similar surface markers and give rise to similar cell types. However, they do have different migration activity *in vitro*. What mechanisms underly the different migration capacity? How this can be used in regenerative medicine?
Authors: Briefly, the difference in the migration activity in the *in vitro* co-culture system can be explained by the fact that DFSC are isolated from a developing tissue (neural crest-derived mesenchymal tissue that forms the dental sac, which surrounds the epithelial enamel organ and the mesenchymal dental papilla of the developing tooth germ before eruption) and might thus exhibit a greater migration activity than DPSC, which belong to a tissue that is already developed and mature.

In the recent literature there are not many articles concerning this topic. In particular, there is a description of receptors and soluble factors involved in the migration but there is not a substantial difference between the two stem cell populations (DPSC and DFSC). To our knowledge, this is the first study on the co-culture of human DPSC and DFSC, thus allowing the investigation of specific/mediated interactions between these two stem cell populations. In fact, we highlighted that their behaviour differs in single population cultures compared with the co-culture assay, which allows an insight on cell kinetics and gene expression modulation. Several recent papers have reported on the migration of DPSC in the presence of different chemoattractants (EMPs) and/or induced migration of DPSC by selective cytokines (e.g. Howard *et al*., 2010; Suzuki *et al*., 2011). However, the migration of DFSC, as well as the migration in a co-culture model, have not been explored in these or other papers. It has been demonstrated that TGFβ1, an important signalling molecule for tissue regeneration and a marker for functional odontoblasts (Melin *et al*., 2000), and SPARC, a non-collagenous protein localised in dentin and bone, are able to stimulate DPSC migration (Pavasant *et al*., 2008). However, these studies report on DPSC migration, but not in DFSC. The role of the various signalling molecules (IGF, EGF, FGF etc) involved in DPSC and DFSC migration is still unknown.

It is well known that *in vivo*, DPSC and DFSC are localised in specific compartments of the tooth organ and their roles are distinct. The *in vitro* interaction between DPSC and DFSC could be useful for better understanding the complex *in vivo* mechanisms involved in repair of specific dental tissues. Few reports hypothesise such interactions between different cell populations of the craniofacial complex. For example, Tancharoen *et al*. (2005) have studied the influence of neuropeptides released from dental pulp cells in the periodontal tissue and suggested a link between periodontitis and pulp inflammation. The results of the study show that DFSC and DPSC in our *in vitro* co-culture system recapitulate the *in vivo* kinetics during odontogenesis, where dental follicle cells surround the dental pulp before the eruption of the tooth. This property of DFSC may be used for the reconstitution of tooth germs *in vitro* or *ex vivo*, thus allowing their implantation into the alveolar bone for the replacement of missing teeth (Mitsiadis and Papagerakis, 2011).

Additional References

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